

# Targeted disruption of hepatic frataxin expression causes impaired mitochondrial function, decreased life span and tumor growth in mice

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**We have disrupted expression of the mitochondrial Friedreich ataxia protein frataxin specifically in murine hepatocytes to generate mice with impaired mitochondrial function and decreased oxidative phosphorylation. These animals have a reduced life span and develop multiple hepatic tumors. Livers also show increased oxidative stress, impaired respiration and reduced ATP levels paralleled by reduced activity of iron–sulfur cluster (Fe/S) containing proteins (ISP), which all leads to increased hepatocyte turnover by promoting both apoptosis and proliferation. Accordingly, phosphorylation of the stress-inducible p38 MAP kinase was found to be specifically impaired following disruption of frataxin. Taken together, these findings indicate that frataxin may act as a mitochondrial tumor suppressor protein in mammals.**

## INTRODUCTION

Mitochondria generate readily available energy equivalents by conversion of macronutrient intermediates into ATP by oxidative phosphorylation (OXPHOS). This process is dependent on iron–sulfur clusters (Fe/S), which are essential parts of mitochondrial enzymes, including aconitase, and complexes I, II and III of the respiratory chain (1).

Friedreich's ataxia is an inherited neurodegenerative disorder (2) caused by reduced expression of the mitochondrial protein frataxin (3) leading to premature death due to cardiac failure (2), diabetes mellitus and insulin resistance (4) and impaired ATP synthesis in muscle of humans (5,6). Concurrently, it was shown that frataxin overexpression promotes ATP synthesis and interacts with the respiratory chain

(7,8). While the primary function of frataxin is still a matter of debate (9), increasing evidence suggests that this protein directs the intramitochondrial synthesis of Fe/S clusters (1,10–12). Individuals suffering from Friedreich ataxia have a reduced life expectancy of 38 years in average (2) and show indications for increased oxidative stress (13–15). Overexpression of frataxin has been shown to reduce intracellular accumulation of reactive oxygen species (ROS) and to prevent menadione-induced malignant transformation of fibroblasts (16). Furthermore, disruption of the *frataxin* homologue in yeast has been shown to cause increased sensitivity against oxidants and promote oxidative damage to both nuclear (17) and mitochondrial DNA (18). In addition, fibroblasts from Friedreich patients exhibit increased sensitivity against ionizing radiation and show an increased frequency of transforming

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events (19). Although cancer is not considered a typical feature of the disorder, Friedreich ataxia patients exhibit various types of malignant or proliferative disorders (20–28).

To evaluate the role of frataxin in liver tissues, we have now disrupted the expression of this protein specifically in hepatocytes of *C57bl6* mice. We here demonstrate the presence and efficacy of the disruption, Fe/S dependent alterations of enzyme activities, decreased OXPHOS and increased ROS formation, decrease of life span, and show formation of multiple liver tumors, paralleled by impaired p38 MAP kinase phosphorylation cumulating in induction of both apoptosis and proliferation. Taken together, the findings indicate that lack of frataxin expression may promote tumor growth in mammals and that frataxin may thus be considered a mitochondrial tumor suppressor protein located upstream of p38 MAP kinase.

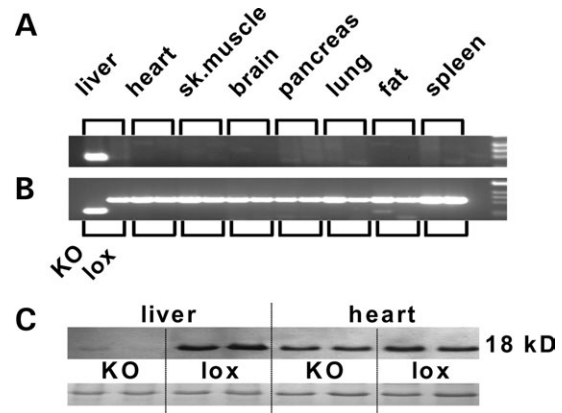
## RESULTS

### Hepatocyte-specific disruption of *frataxin* expression

Frataxin is a nuclear encoded protein with an N-terminal signal targeting the protein to the mitochondrial compartment (2). We have used the *cre/loxP* system to remove exon 4 of the *frataxin* gene in a tissue-specific manner as previously described (11,29) except for hepatocyte-specific expression of Cre recombinase was obtained by using mice carrying an *albumin* promoter-driven *Cre* transgene (30). Presence, efficiency and specificity of disruption were shown at genomic, transcriptional and translational levels by using genomic PCR by primers flanking the *loxP* sites of the targeting allele (11) (Fig. 1A), reversely transcribed PCR with primers located in exons 3 and 5 of the *frataxin* gene (Fig. 1B) and immunoblotting against murine frataxin protein (Fig. 1C). The findings so far indicate that disruption of *frataxin* expression is restricted to liver specimen from knockout mice (Fig. 1A–C) and that disruption efficacy is almost complete (Fig. 1C), because faint remnant signals (Fig. 1C, left lane) are likely due to non-hepatocyte cells contained in the liver samples.

### Decreased life span and liver tumor formation in *frataxin* knockout mice

Although knockout mice were born in the expected Mendelian frequency (data not shown) ( $P = 0.7351$ ) and had normal body weight (Fig. 2A), they subsequently failed to thrive, as indicated by a lack of weight gain (Fig. 2A) and a specific reduction of body fat as determined by nuclear magnetic resonance (data not shown). To investigate whether liver function might be impaired in knockout mice, serum levels for albumin (Alb), cholinesterase (ChE), L-alanine transferase (ALAT) and lactate dehydrogenase (LDH) were determined (Fig. 2B–E). The first two parameters (Alb and ChE) are commonly used to quantify synthesis capacity of hepatocytes and were found to be moderately but significantly reduced (Fig. 2B and C). Two other parameters to quantify putative hepatocyte damage were determined (ALAT and LDH) and were found to be mildly increased (Fig. 2D and E), suggesting continuous damage to hepatocytes. Nevertheless, changes were found to



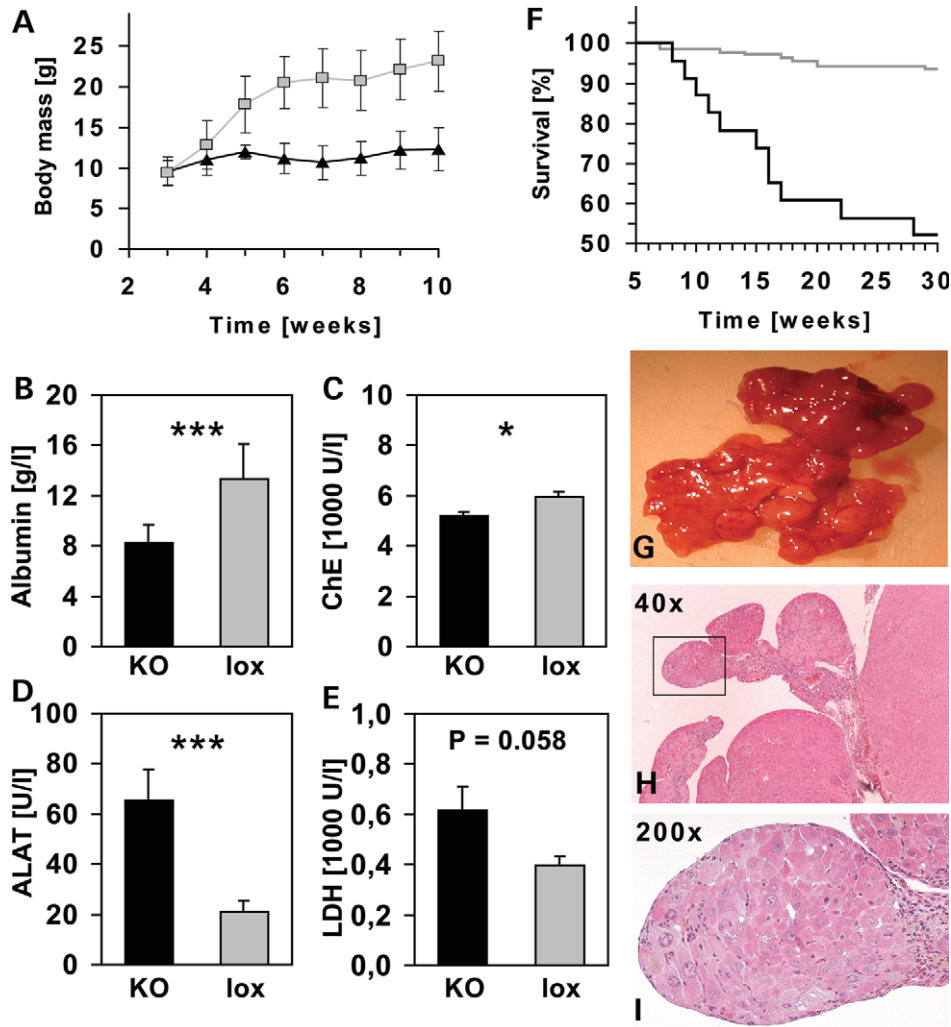
**Figure 1.** Efficacy and specificity of hepatocyte-specific *frataxin* knockout in mice. (A) Detection of the disrupted *frataxin* allele by PCR amplification of genomic DNA. (B) Detection of a missing exon 4 within *frataxin* cDNA by reversely transcribed PCR in liver RNA samples of knockout mice. In (C) immunoblots against murine frataxin protein (18 kDa) and a Ponceau red stain of the corresponding blot (loading control, also applies to Fig. 5C and E) are displayed.

be comparably moderate but may be sufficient to contribute to the failure to thrive leading to hepatic cachexia (31).

Furthermore, knockout mice exhibited a significantly decreased life expectancy (Fig. 2F) leading to reduction of the number of knockout animals by almost 50% at an age of 30 weeks after birth. Whereas wild-type *C57bl6* mice are known to be susceptible to liver tumor development at senescence only, anatomical evaluation of young knockout mice revealed the presence of multiple liver tumors (Fig. 2G), which were not observed in age-matched control animals. The average number of macroscopically visible tumors per animal was 32.4 (+15.7,  $P =$  not applicable, as number of tumors in control group equaled zero). In these tumors, a highly increased number of mitotic and apoptotic hepatocytes as well as numerous polyploid and poorly differentiated cells were observed (Fig. 2H and I).

### Increased oxidative stress in *frataxin* knockout mice

Previously published evidence suggests an increase of oxidative stress or ROS formation in fibroblasts or blood samples from Friedreich ataxia patients (13–15). Therefore, we questioned whether oxidative stress might be elevated in livers of *frataxin* knockout mice. First, we quantified a marker for lipid oxidation, so-called thiobarbituric-acid reactive substances (TBARS), which we found to be significantly elevated in liver specimen from *frataxin* knockout mice (Fig. 3A). Subsequently, levels of reduced and oxidized glutathione in such specimen were determined. Glutathione in its reduced state (GSH) confers to the quantitatively most important buffering system against oxidative stress in mammals. Consistently, reduced glutathione levels have been found diminished in blood samples of Friedreich ataxia patients (14) and have been described to be elevated in murine fibroblasts overexpressing human frataxin (16). In liver specimen of our knockout mice, levels of oxidized glutathione (GSSG) were found to be significantly elevated (Fig. 3B), whereas



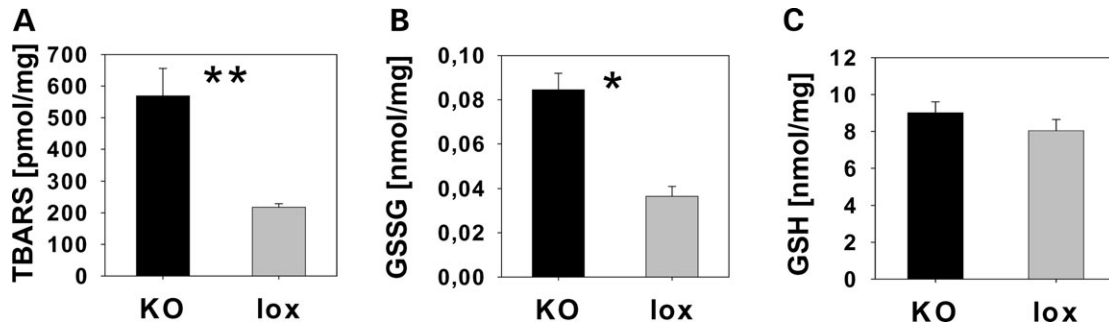
**Figure 2.** Decreased life span and liver tumor formation due to *frataxin* disruption. (A) Body weight gain in offspring starting at 3 weeks of age (squares indicate control animals, triangles indicate knockout animals). (B–E) Serum levels for albumin (B), cholinesterase (C), L-alanine transferase (D) and lactate dehydrogenase (E) in *frataxin* knockout mice (black) and control genotypes (gray) (both apply to all subsequent figures). (F) Kaplan–Meier survival graph of knockout mice (black line) versus control genotypes (gray line). (G–I) A typical liver specimen of hepatocyte-specific *frataxin* knockout mice; original enlargement: (G) none; (H) 40-fold and (I) 200-fold. \*Indicates  $P < 0.05$ ; \*\*indicates  $P < 0.005$ ; \*\*\*indicates  $P < 0.0005$ .

levels of reduced glutathione were not found to be affected by disruption of *frataxin* expression (Fig. 3C). Taken together, these findings suggest that a detectable increase in oxidative stress occurs in liver specimen of knockout mice, although the overall buffering capacity against ROS remains unaffected as reflected by unaltered levels of reduced glutathione.

#### Reduction of hepatic mitochondrial function in *frataxin* knockout mice

Disruption of mitochondrial proteins may reduce the number of mitochondria per cell. Therefore, we quantified mitochondrial marker proteins including cytochrome C (described subsequently) (Fig. 5E) as well as mitochondrial DNA (mtDNA) content by Southern blotting using the mitochondrially encoded subunit III of cytochrome oxidase as a probe (mtCOXIII, Fig. 4A, upper panel). After normalization of

the mtCOXIII signal to 18S rDNA (Fig. 4A, lower panel), no significant difference in mtDNA content was observed when knockout and control animals were compared ( $P = 0.501$ ). Subsequent quantification of enzymes within the Krebs cycle as well as the respiratory chain indicated that lack of *frataxin* indeed selectively affects activities of those proteins containing Fe/S clusters, including aconitase, and complexes I, II and III of the respiratory chain (Fig. 4B). Concurrently, activity of fumarate hydratase, an enzyme functioning independently of Fe/S clusters, was found to be unaffected (Fig. 4B). Taken together, these findings suggest an impairment of Krebs cycle flux, which should lead to decreased oxidative capacity and ultimately an energy deficit within affected cells. Hence, we quantified oxygen consumption, which was found to be reduced in liver specimen of *frataxin* knockout mice when compared with control animals (Fig. 4C), whereas other tissues of



**Figure 3.** Increased markers of oxidative stress following *frataxin* disruption. (A) Amounts of thiobarbituric reactive substances (TBARS) in liver specimen of *frataxin* knockout mice and control genotypes. Error bars indicate standard deviations (applies to all subsequent figures). (B) Levels of oxidized glutathione and (C) levels of reduced glutathione in liver specimen. \*Indicates  $P < 0.05$ ; \*\*indicates  $P < 0.005$ ; \*\*\*indicates  $P < 0.0005$ .

knockout animals showed a respiratory activity comparable to that of controls (data not shown). Subsequent quantification of ATP levels revealed a remarkable reduction in liver tissues of knockout animals (Fig. 4D), whereas other tissues of knockout animals contained normal amounts of ATP in comparison to control animals (data not shown). Taken together, these findings suggest that disruption of *frataxin* causes a specific impairment of Fe/S cluster containing mitochondrial enzymes leading to an impairment of respiration and ATP synthesis, consistent with previously published findings regarding Fe/S enzyme activity (10–12) and OXPHOS (5,7,8) in states of altered frataxin expression.

#### Increased apoptosis in hepatocytes of *frataxin* knockout mice

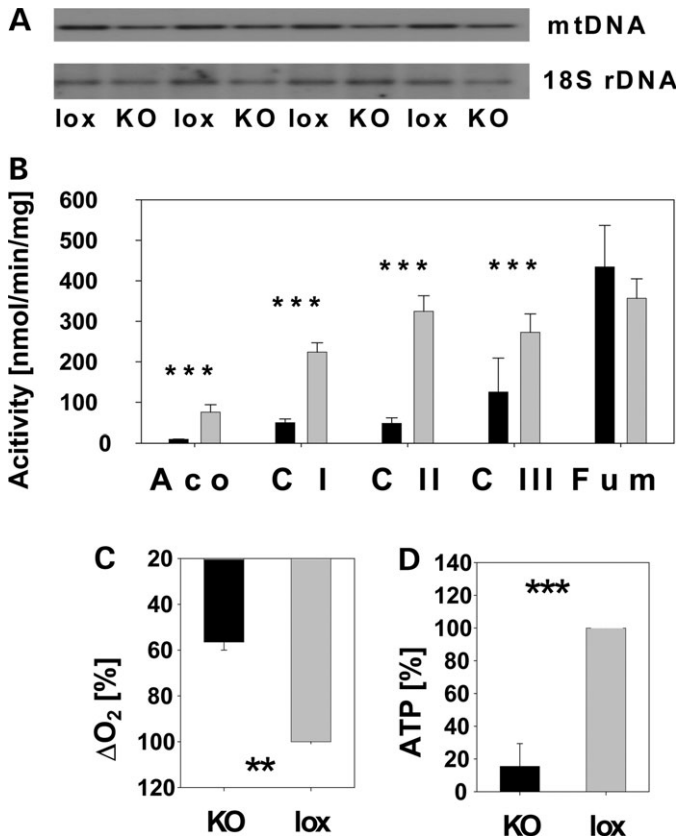
Impaired OXPHOS, due to disruption of *frataxin* as well as other reasons, causes depletion of intracellular ATP (Fig. 4D). Chronic depletion of ATP as well as increased ROS (see earlier) may cause programmed cell death by the activation of previously established molecular pathways. Translocation of the pro-apoptotic protein Bax to the mitochondria is an early event during apoptosis in eukaryotic cells. Accordingly, in hepatic tissue lysates lacking frataxin (Fig. 5A), we observed an increase in the expression of Bax (Fig. 5B) as well as a translocation of Bax to the mitochondrial fractions of tissue lysates (Fig. 5C). A subsequent release of cytochrome C from the mitochondria to the cytosol was consistently observed (Fig. 5D), whereas the content of membrane associated cytochrome C in the mitochondrial fractions was found unaltered (Fig. 5E), again suggesting a normal amount of mitochondria in *frataxin* knockout hepatocytes (Fig. 4A). Cleavage of caspase 3 into its active form reflects a terminal stage of the pro-apoptotic program. Accordingly, only hepatocytes of knockout animals showed detectable level of activated caspase 3 protein (Fig. 5F). To validate these findings by an independent method, TUNEL stains, reflecting apoptotic cells, were performed. This assay also showed an increased number of apoptotic events in sections of *frataxin* knockout livers ( $P < 0.01$ ) (Fig. 5G). Of note, no activation of pro-apoptotic p53 was observed (data not shown), consistent with a previously described activation of Bax independent

of p53, e.g. by arsenic trioxide (32), a substance that interestingly functions as an inhibitor of mitochondrial Krebs cycle activity. Taken together, these findings demonstrate an induction of apoptotic pathways in frataxin-deficient hepatocytes.

#### Increased proliferation in hepatocytes of *frataxin* knockout mice

As an increased frequency of apoptotic events alone is not sufficient to explain neither tumor formation nor tumor growth and as increased levels of ROS may cause tumor formation and/or growth, we asked whether increased apoptotic events were paralleled by an induction of pro-proliferative molecular pathways in livers of *frataxin* knockout mice, together potentially causing an increased hepatocyte turnover. Therefore, we first quantified two potentially oncogenic members of the family of heat shock proteins, HSP70 and HSP25, the latter being the murine homologue of human HSP27. While expression of HSP70 was found to be decreased in liver specimen from *frataxin* knockout mice (Fig. 5H), protein levels of HSP25 were increased (Fig. 5I). Regarding impaired OXPHOS activity (Fig. 4C and D), it should be noted that the activation of HSP70 is known to be an ATP-dependent process, whereas induction of HSP27, and hence presumably also HSP25, occurs independently of ATP (33).

Next, we quantified expression levels and phosphorylation status of the three major members of the mitogen-activated protein kinase family, p44/42, SAPK/JNK and p38 MAP kinase. Whereas no change in expression or phosphorylation of p44/42 or SAPK/JNK was detected (data not shown), phosphorylation of p38 MAP kinase was found to be impaired in knockout liver specimen (Fig. 5J), while basal p38 expression remained unaltered (Fig. 5K). The MAP kinase p38 in its phosphorylated state functions as a tumor suppressor protein (34–36), specifically in liver (37,38) and has been shown to suppress growth by inhibition of cyclin D1/cyclin-dependent kinase 4 (cdk4) complexes (34). We subsequently quantified cdk4 expression, which we found to be increased in knockout specimen (Fig. 5L) suggesting promotion of G1 to S transition of the cell cycle, consistent with a persistent pro-proliferative stimulus. Concurrently, immunostaining with an antibody against Ki-67 protein, a marker for proliferating cells, revealed

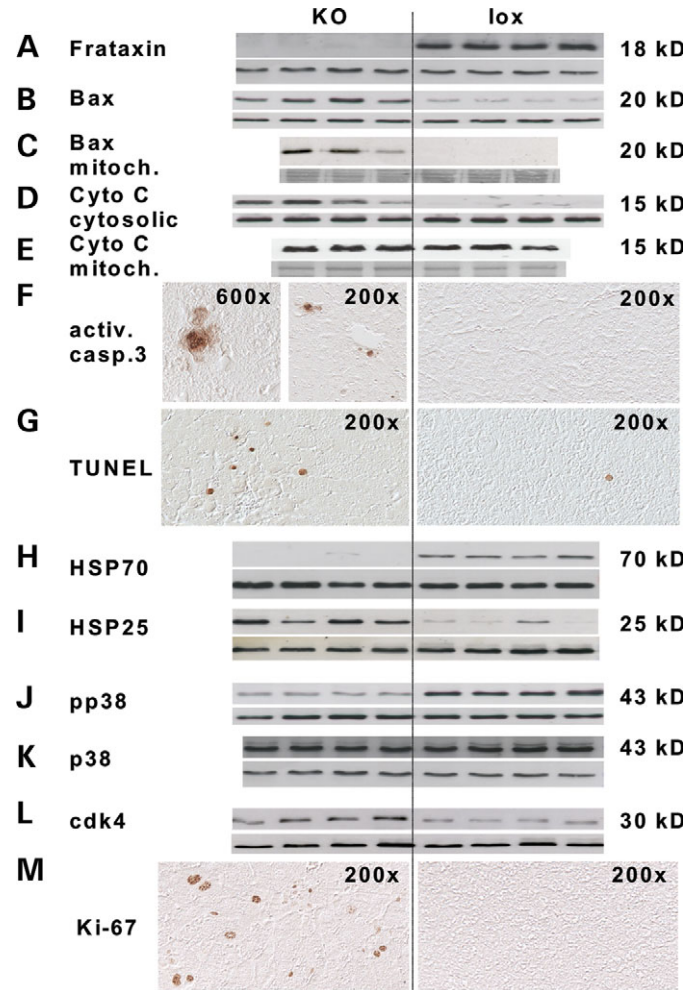


**Figure 4.** Impaired mitochondrial function due to *frataxin* disruption. (A) Southern blotting of mtDNA of liver samples from *frataxin* knockout mice and control genotypes employing an mtCOXIII probe; below the corresponding loading control (18S rDNA). (B) Specific activities of mitochondrial enzymes in liver samples from *frataxin* knockout mice and control genotypes. Abbreviations: Aco, aconitase; C I, complex I; C II, complex II; C III, complex III and Fum, fumarate hydratase. (C) Oxygen consumption of liver specimen from *frataxin* knockout mice and control genotypes. (D) ATP content of liver specimen from *frataxin* knockout mice and control genotypes. \*Indicates  $P < 0.05$ ; \*\*indicates  $P < 0.005$ ; \*\*\*indicates  $P < 0.0005$ .

a significant increase in the number of cells about to divide in knockout animals when compared with liver sections from control genotypes (Fig. 5M) ( $P < 0.00001$ ). Taken together, these findings suggest that the disruption of *frataxin* causes a reduction in OXPHOS and an increase in ROS formation, impaired phosphorylation of p38, and increased expression of cdk4, leading to increased proliferation of knockout hepatocytes.

## DISCUSSION

By disruption of *frataxin* in murine hepatocytes, we here show impaired mitochondrial function, decreased life span, and, unexpectedly, formation of tumors in knockout mice. Consistent with previously published data, we and others have shown that *frataxin* controls mitochondrial function and ATP synthesis (5–8,10), as suggested by one of its proposed primary functions, the control of Fe/S cluster synthesis (1,12). Secondly, disruption of *frataxin* causes increased formation of ROS, as indicated by elevated levels of TBARS and oxidized glutathione (GSSG) in liver specimen from knockout mice,



**Figure 5.** *Frataxin* disruption promotes both apoptosis and proliferation. (A) Immunoblots against murine frataxin (upper) and a subsequent re-blot against  $\alpha$ -tubulin (lower, loading control) (the latter also applies to B, D, H–L) in whole cell lysates. (B) Immunoblot against Bax in whole cell lysates and (C) immunoblot against Bax in mitochondrial fractions of whole cell lysates. (D) Immunoblot against cytochrome C in cytosolic fractions of whole cell lysates. (E) Immunoblot against cytochrome C in mitochondrial fractions of whole cell lysates. (F) A typical stain against activated/cleaved caspase 3 and (G) a typical TUNEL stain, both on sections from *frataxin* knockout mice (left) and control genotypes (right); original enlargement 200-fold, except otherwise indicated. (H) An immunoblot in whole cell lysates against heat shock protein 70 (HSP70) and (I) against HSP25. (J) Immunoblot against phosphorylated p38 MAP kinase in liver samples from *frataxin* knockout mice, (K) a blot against basal p38 MAP kinase in these samples and (L) immunoblot against cyclin-dependent kinase 4. (M) A typical immunostaining against Ki-67 on sections from *frataxin* knockout mice (left) and control genotypes (right); original enlargement 200-fold.

and consistent with previously published findings (13–16). Nevertheless, buffering capacity against ROS remained unaffected as indicated by unaltered levels of reduced glutathione (GSH), owing some support to recent data suggesting a rather limited role of oxidative stress in the development of the Friedreich ataxia phenotype (39). Thirdly, we observed reduction of life span in affected mice consistent with previously published data on extension of life span in eukaryotes with

an increase in mitochondrial respiration (40) as well as reduced life expectancy due to increased ROS formation in mice (41). Finally, we found that frataxin deficiency promotes tumor formation in mice by impairing activation of the tumor suppressor p38 MAP kinase (34–36), which has been found to be important for growth and tumorigenesis especially in liver (37,38). Specifically, deficiency of frataxin leads to an enhanced hepatocyte turnover by simultaneous induction of both apoptosis, which is typically observed following depletion of ATP and occasionally observed following induction of ROS, as well as proliferation, which may be induced by impaired phosphorylation of p38 MAP kinase and possibly increased formation of ROS. Further experiments employing liver-specific frataxin knockouts as well as inbred, genetically unmodified mice will have to show whether reduction of Fe/S-dependent enzymes and OXPHOS is sufficient to impair phosphorylation of p38 MAP kinase, especially as ROS are known to typically induce rather than impair activity of stress kinases (42), and specifically p38 MAP kinase (42).

Numerous cancer specimen exhibit mtDNA deletions, reduced mitochondrial content, altered mitochondrial morphology and impaired oxidative capacity (43–45) as well as an increase in glycolytic rate and lactate production (46,47). Consistently, disorders of the respiratory chain predispose to hepatocellular carcinoma in humans (48), and rare inherited deficiencies of mitochondrial succinate dehydrogenase subunits or mitochondrial fumarate hydratase can cause tumors in humans (49). In this regard, it has been predicted that these inherited deficiencies should cause increased formation of ROS in parallel with impaired OXPHOS, hypothetically culminating in both increased apoptosis and proliferation (49). By using our mouse model of impaired OXPHOS due to depletion of frataxin, we here confirm this hypothesis. However, although oxidative stress is observed in frataxin-deficient liver specimen, a primary role of ROS in tumor formation remains a matter of debate as (i) the buffering capacity of knockout hepatocytes against ROS was found to be unaltered and (ii) the tumor suppressor p38 was found less phosphorylated in *frataxin* knockout animals than in control mice, while significant levels of ROS typically induce p38 activity (42). Further experiments employing transformed cell lines overexpressing frataxin may be both useful and required to dissect the concurrent roles of impaired OXPHOS and increased ROS formation in frataxin-dependent induction of tumor growth and are currently underway.

Friedreich ataxia is a disease predisposing to occasional tumors at young age (20–28); nevertheless, malignant disorders are not considered a mandatory complication of the disease. As previously discussed for a similar apparent inconsistency in fumarate hydratase deficient individuals (50), Friedreich ataxia patients typically exhibit a decreased life expectancy of 38 years on average, which may prevent tumors to evolve into a clinically visible state. Furthermore, while Friedreich ataxia patients exhibit reduced, albeit detectable, levels of frataxin protein in their tissues (2), knockout mice including our liver-specific animals have undetectable expression levels, potentially accelerating the phenotype, as previously discussed for the disruption of frataxin expression in tissues other than liver (29). Hence, the phenotype observed here is probably induced by complete disruption of frataxin

expression, while Friedreich ataxia patients (as well as their heterozygous relatives) may exhibit an increased risk for malignancies only if they obtain a normal life span, which should be taken into account as soon as successful treatments for the disease have been established. Furthermore, prospective studies to determine the cancer risk in first-degree relatives of Friedreich ataxia patients might be useful to further test this hypothesis.

In summary, we here have shown that lack of hepatic frataxin expression causes liver tumor growth in mice following impaired mitochondrial function and increased ROS formation and that this unprecedented effect of a mitochondrial protein may be mediated by modulating the activity of p38 MAP kinase. Hence, frataxin might be considered a metabolic tumor suppressor protein located upstream of established stress kinases in mammals.

## METHODS AND MATERIALS

### Generation of knockout mice

Animals were generated (11), bred (29) and maintained (29) as described before except for beta-cell specific *Ins2-cre* mice were replaced by hepatocyte-specific *Alb-cre* (30) animals, which were 67% *C57bl6* and 33% *FVB* of origin, whereas *frataxin loxP* animals were at least 90% of *C57bl6* origin. Genotyping and detection of knockout animals at genomic and transcriptional levels were previously described (29). Detection of knockout at translational levels was performed with a polyclonal antibody against mouse frataxin (11) by immunoblotting as described (16).

### Histology and immunohistochemistry

Methods have been described before (29), except for TUNEL assays were performed by using a TACS XL Apoptosis Detection Kit (Trevigen, Gaithersburg, MD, USA).

### Southern blotting

Methods have been described before (51) except for *NheI* (Roche, Basel, Switzerland) was used for enzymatic restriction of murine DNA prior to gel electrophoresis.

### Metabolic and enzymatic assays

Mitochondrial enzyme activities were determined as described before (52). Oxygen consumptions, ATP contents and mitochondrial membrane potentials were measured as previously described (7). Measurements of TBARS were performed as previously described (53). Quantification of oxidized and reduced glutathione was performed as previously described (54). Serum levels for albumin, ALAT, ChE and LDH were thankfully determined by the clinical laboratory of the German Institute for Human Nutrition employing standard assays. All assays were performed in samples derived from at least four animals per genotype.

## Signal transduction

Immunoblots were performed as described before (16) except for additional polyclonal antibodies against Bax, basal p38, Thr180/Tyr182 phosphorylated p38, basal p44/42, Thr202/Tyr204 phosphorylated p44/42, basal SAPK/JNK, and Thr183/Tyr185 phosphorylated SAPK/JNK (Cell Signalling, Beverly, MA, USA), basal p53 (Novo-Castra Laboratories, Newcastle upon Tyne, UK), basal p53 (Exalpha Biologicals, Boston, MA, USA), HSP25 (Stressgen, Victoria, BC, Canada), and additional monoclonal antibodies against cdk4, Ser15 phosphorylated p53, and basal p53 (Cell Signalling), cytochrome C (BD Biosciences, Franklin Lakes, NJ, USA), HSP70 (Stressgen) and  $\alpha$ -tubulin (Sigma-Aldrich) were used, and phosphatase inhibitors (*Complete*, Roche) were added whenever applicable.

## Statistical analyses

Methods have been described before (29).

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*Conflict of Interest statement.* None declared.

## NOTE ADDED IN PROOF

A corresponding manuscript describing inhibitory effects of frataxin on cancer growth was accepted for publication at the Journal of Biological Chemistry (digital object identifier: 10.1074/jbc.M511064200, <http://www.jbc.org/cgi/doi/10.1074/jbc.M511064200>).

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## **CORRIGENDUM**

# **Targeted disruption of hepatic frataxin expression causes impaired mitochondrial function, decreased life span and tumor growth in mice**

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